An endogenously deposited fibrin scaffold determines construct size in the surgically created arteriovenous loop chamber model of tissue engineering

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Background: **An arteriovenous loop (AVL) enclosed in a polycarbonate chamber in vivo, produces a fibrin exudate which acts as a provisional matrix for the development of a tissue engineered microcirculatory network.**
Objectives: **By administering enoxaparin sodium - an inhibitor of fibrin polymerization, the significance of fibrin scaffold formation on AVL construct size (including the AVL, fibrin scaffold, and new tissue growth into the fibrin), growth, and vascularization were assessed and compared to controls.**
Methods: **In Sprague Dawley rats, an AVL was created on femoral vessels and inserted into a polycarbonate chamber in the groin in 3 control groups (Series I) and 3 experimental groups (Series II). Two hours before surgery and 6 hours post-surgery, saline (Series I) or enoxaparin sodium (0.6 mg/kg, Series II) was administered intra-peritoneally. Thereafter, the rats were injected daily with saline (Series I) or enoxaparin sodium (1.5 mg/kg, Series II) until construct retrieval at 3, 10, or 21 days. The retrieved constructs underwent weight and volume measurements, and morphologic/morphometric analysis of new tissue components.**
Results: **Enoxaparin sodium treatment resulted in the development of smaller AVL constructs at 3, 10, and 21 days. Construct weight and volume were significantly reduced at 10 days (control weight 0.337 ± 0.016 g [Mean ± SEM] vs treated 0.228 ± 0.048, [$P < .001$]: control volume 0.317 ± 0.015 mL vs treated 0.184 ± 0.039 mL [$P < .01$]) and 21 days (control weight 0.306 ± 0.053 g vs treated 0.198 ± 0.043 g [$P < .01$]: control volume 0.285 ± 0.047 mL vs treated 0.148 ± 0.041 mL, [$P < .01$]). Angiogenesis was delayed in the enoxaparin sodium-treated constructs with the absolute vascular volume significantly decreased at 10 days (control vascular volume 0.029 ± 0.03 mL vs treated 0.012 ± 0.002 mL [$P < .05$]).**
Conclusion: **In this in vivo tissue engineering model, endogenous, extra-vascularly deposited fibrin volume determines construct size and vascular growth in the first 3 weeks and is, therefore, critical to full construct development. (J Vasc Surg 2008;48:974-85.)**

Clinical Relevance: **Tissue engineering is an expanding field and ultimately has the potential of growing new organs and tissues for replacement of damaged or lost organs. The Bernard O'Brien Institute of Microsurgery (Melbourne, Australia) has developed an in vivo model in which a surgically created arteriovenous loop (AVL) created on the rat femoral vessels and placed in an empty poly-carbonate chamber under the groin skin exudes a fibrin matrix that fills the chamber in the first day. Capillaries begin sprouting from the femoral vein portion of the AVL 3-5 days later, supported by the fibrin matrix (Lokmic et al).[5] The chamber is filled to about three-quarters full with vascularized connective tissue in about 3 weeks. The endogenous fibrin scaffold is an integral part of the construct. Many tissue engineering models use biological or synthetic scaffolds to support and maintain the construct. This study looks at the significance of this endogenous fibrin scaffold by partially inhibiting its formation using enoxaparin sodium (an inhibitor of fibrin polymerization) and subsequently comparing with untreated controls, construct weight, and volume and vascularization over the following 3 weeks. Utilizing endogenous fibrin scaffolds has enormous potential for in vivo tissue engineering, particularly in growing large three dimensional vascularized tissues. Endogenous fibrin scaffolds are biologically compatible with the host and will not provoke an inflammatory or foreign body response. This study explores the significance of this natural scaffold in determining construct size and vascularization in an in vivo model of tissue engineering.**

Tissue engineering and regenerative medicine are emergent fields of medicine that will have broad applications in the management of disease and trauma. The technologies associated with these fields are multiple and include molecular and cell biology, especially stem cell biology, bioengineering, and transplantation surgery and immunology. Despite tissue engineering's great promises, most of the advances so far have been in the laboratory and the leap to in vivo human clinical

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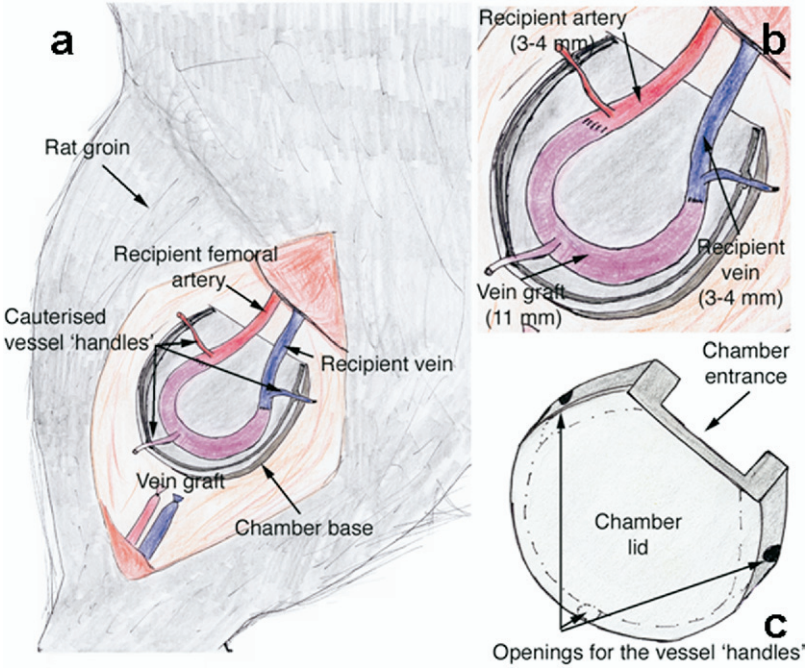

Fig 1. a, Diagram of the arteriovenous loop (recipient femoral artery, vein and vein graft) positioned on the chamber base in the rat groin. **b,** Diagram illustrating the dimensions of the components of the AVL on the chamber base in the groin. **c,** Diagram of the chamber lid. (Illustrations by Dr Zerina Lokmic).

application requires the engineered product to survive implantation into the body. Cytoprotective therapies will complement this process, but the key to survival of implanted cells and tissues is rapid vascularization. We have developed a chamber for this purpose that is implanted subdermally together with an arteriovenous fistula inset within the chamber's hollow interior. This configuration leads to a rapid angiogenic burst from the fistulous loop that progressively fills the chamber space. The proliferating and expanding vascularized connective tissue is an ideal "bed" for the vascularization of seeded cells and small tissue pieces. Using this approach we have been able to grow heart tissue from neonatal cardiomyocytes[1] and adipose tissue from implanted muscle pieces.[2] There is a need for improving the implantation efficacy of many current cell seeding therapies. Vascularization is the common denominator of their success.

The key intermediary to facilitate the delivery of laboratory products for tissue engineering procedures will be the vascular surgeon. He/she will critically create the vascular environment that nourishes the product and ensures maximum cell survival, whether this be an arteriovenous fistula, pedicle, or some other novel vascular bed. The vascular surgeon should be at the forefront of this technology, as advisor to the bioengineer and cell biologist, as the professional who will biopsy tissues/cells for laboratory manipulation, and as the surgeon to create the vascular bed and implant the chamber and the manipulated cells or tissue.

This study addresses an aspect of the angiogenic proliferation from the arteriovenous loop (AVL) in our chamber and highlights the efficacy of fibrin as a positive influence on tissue growth in the AVL chamber environment.

To establish new tissue growth in vivo for tissue engineering purposes, a tissue engineering 'construct' is created. The construct will include a scaffold that mimics the extra cellular matrix of the tissue or organ being grown, and usually a specific implanted cell population and/or a vascular supply. The scaffold, which may be either synthetically or biologically derived, has a number of purposes including the maintenance of construct size and support of implanted cells and angiogenic growth.[3] Numerous scaffolds have been developed for tissue engineering purposes with a variable degree of success, however, many of these scaffolds generate foreign body responses in the developing tissue.[4]

In the AVL chamber model of tissue engineering, a vein graft is microsurgically inserted between the cut ends of the femoral artery and vein (Fig 1). The AVL is inserted in a polycarbonate chamber without the addition of growth factors or an exogenous scaffold. The microsurgical procedure leads to the deposition within the first day (Dr A Messina, personnel communication) of a fibrinogen-based exudate largely exuded from the anastomosis, that is protected in vivo by the walls of the polycarbonate chamber.[5] Observations of the AVL without the chamber at the same site indicate dissipation of the fibrin exudate if the AVL is not contained within a chamber, and complete inhibition of new tissue growth around the AVL if it is not contained in a chamber (Kelly, JL, Messina A, manuscript in preparation).

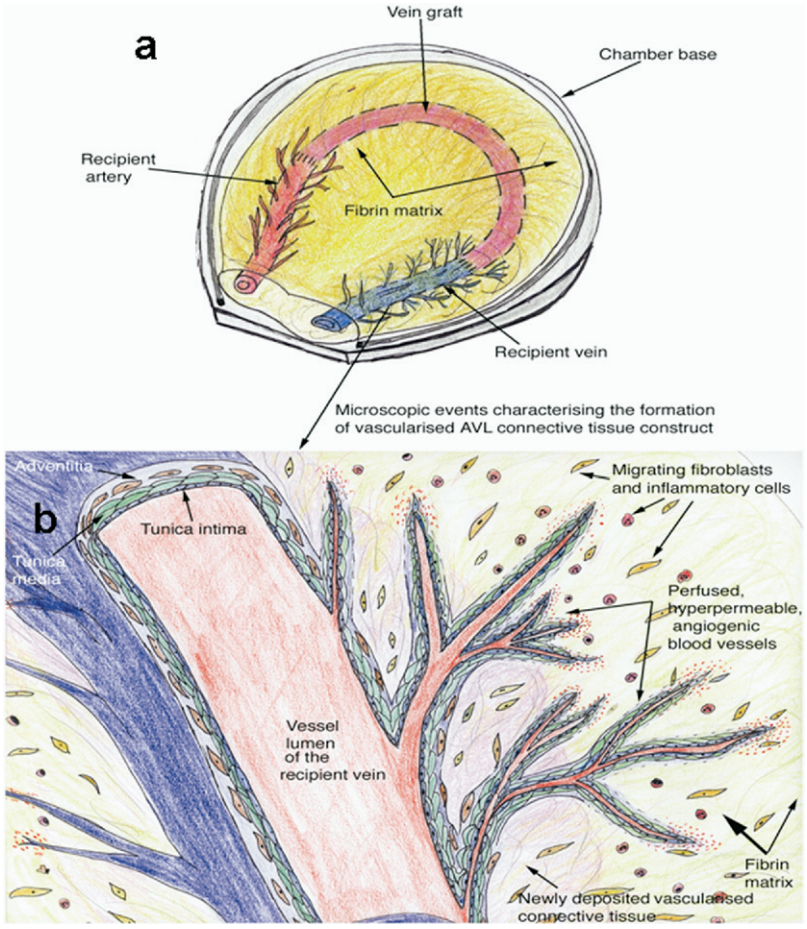

Fig 2. a, Diagram of the AVL on the chamber base at approximately 7-10 days, illustrating the fibrin scaffold that almost fills the chamber and sprouting from the recipient femoral artery and vein. **b,** Diagram illustrating in detail the early migration of cells into the fibrin scaffold, and sprouting of capillaries from the recipient vein into the fibrin scaffold. (Illustrations by Dr Zerina Lokmic).

Within 3 days of implantation, blood borne and vascular wall derived mesenchymal and endothelial precursors migrate into this fibrin scaffold largely from the recipient vein and by 7 days a cuff of new capillaries supported by connective tissue deposition sprout off the AVL,[5] (Fig 2). Angiogenic sprouting from the AVL is absolutely essential to the creation of an "engineered" microcirculation in the AVL construct. (In terms of the AVL chamber model the term 'construct' refers to the chamber contents, that is, the AVL and surrounding fibrin scaffold and new tissue [blood vessels and connective tissue] which may have grown into the scaffold. The term construct does not include the chamber itself).

The new tissue that grows into the fibrin scaffold is derived from migrating cells that move from the blood stream through the wall of the AVL and into the fibrin. The chamber walls largely prohibit interaction with the outside tissue. This has distinct advantages for the AVL construct, as the chamber walls protect the fibrin scaffold in the first few days and keep it intact.

The result is a highly vascularized construct composed of a complex microcirculatory network including arterioles and venules connected by the AVL to the systemic circulation. Capillary sprouting off the AVL is an advantage as this directly connects the new microcirculation to the systemic circulation and means that as the new microcirculation arises from a clearly defined vascular pedicle it can be readily surgically transplanted to other sites.

Interestingly, angiogenesis (development of the new vessels from pre-existing vessels) appeared to cease once the edge of the fibrin scaffold was reached by the sprouting capillaries[5] suggesting that the volume of fibrin scaffold may exert limitations on construct growth.

The role of fibrin in angiogenesis is best observed during wound healing. Here the endogenously deposited fibrin is a polymerized scaffold containing growth factors and cytokines which enable migration of inflammatory cells, myofibroblasts, and angiogenic capillaries.[6,7] Angiogenic growth factors - vascular endothelial growth factor (VEGF)[6] and fibroblast growth factor-2 (FGF-2)[7] can bind

to fibrinogen with high affinity. In addition, sprouting capillaries in the tissue adjacent to the deposited fibrin commence development with the proteolysis of the pre-existing basement membrane[8] and proliferation of migrating endothelial cells under the influence of VEGF, FGF-2, and transforming growth factor-β (TFG- β).[8,9] The proliferating capillary sprouts migrate into the fibrin eventually becoming functional blood vessels. This same early cell-supportive role of endogenously deposited fibrin scaffold was observed in the formation of surgically created rat AVL tissue engineering constructs.[5]

The hypothesis of this study is that chronic administration of enoxaparin sodium will inhibit fibrin and vascularized tissue formation inside experimental subcutaneous polycarbonate chambers containing a surgically-created AVL.

The aim of this study was to examine the significance of endogenously deposited fibrin scaffold in the tissue and microvascular development of the AVL construct by inhibiting the fibrin polymerization, and, therefore, deposition using the low molecular weight heparin, enoxaparin sodium. Separate groups of animals (n = 6/timepoint) in the control (saline-treated) and experimental series were examined at 3, 10, and 21 days for construct weight and volume, morphology and morphometric analysis of tissue components: fibrin, connective tissue, and blood vessels. The selection of these time points was based on a previous study where, in the absence of exogenously added scaffolds or growth factors, the peak of construct weight and volume was observed at 7 days while the peak percent vascular volume was observed at 10 days.[5]

METHODS AND MATERIALS

Animals. Six male Sprague-Dawley rats (ARC, Perth, Western Australia) weighing 280-320 grams were used for each time point (3, 10, and 21 days) in the control and experimental series. All experimental procedures were approved by the Animal Ethics Committee of St Vincent's Hospital, Melbourne, Australia, and were conducted in accordance with the Australian National Health and Medical Research Council guidelines for the care and maintenance of animals.

Pre-operative treatment. Enoxaparin sodium binds to anti-thrombin III leading to inhibition of thrombin generation, and inhibition of Factor Xa, as well as induction of a sustained release of tissue factor pathway inhibitor.[10] The end point of enoxaparin sodium treatment is the inhibition of fibrin polymerization within the circulatory system. For this reason, enoxaparin sodium is used in pre-operative and post-operative care of patients undergoing surgery to prevent post-operative thrombotic complications. The pre-operative doses used in human patients in clinical settings are 0.3 mg/kg weight (for patients with low risk of developing thrombosis), 0.6 mg/kg (for patients with high risk of developing thrombosis), and a post-operative dose 1.5 mg/kg.[11-13]

Therapeutic doses of enoxaparin sodium reported in the literature for animal experimentation vary widely.[14-17] An increased occurrence of hemorrhage has been reported when doses greater than 4 mg/kg are used.[10] Other studies indicate that in rats, enoxaparin sodium can be administered at the same doses as in human subjects.[18-20] In this study, the experimental rats (Series II) were injected intraperitoneally with 0.6 mg/kg enoxaparin sodium (Aventis Pharma Pty Ltd, Paris, France) 2 hours prior to surgery while control rats (Series I) received an equivalent volume of drug vehicle, normal saline. No adverse signs of hemorrhage were subsequently seen in any enoxaparin sodium-treated rats.

Our previous studies indicate that the fibrin accumulation is an immediate process commencing as the AVL conducts a blood flow, (day 1) and continues to a lesser extent (days 5-21) through the action of "leaky" new capillaries. The pre-operative and 6-hour post-operative dose will prevent the immediate deposition (day 1). The daily post-operative administration aims to reduce further deposition from "leaky" capillaries that are just forming (days 5-21).

Creation of the AVL chamber model. The rats were anesthetized by intraperitoneal injection of phenobarbitone (30 mg/kg), and an AVL surgically created by interposing an 11 mm vein graft harvested from the left femoral vein, between the proximal stumps of the divided right femoral artery and vein as previously described[5] (Fig 1, *a, b*). A sterile non-porous polycarbonate chamber base was placed under the AVL (approximately 3-4 mm of recipient artery and vein were inside the chamber) and the base sutured in position with 6.0 nylon sutures. The AVL was positioned in the chamber using the cut superficial inferior epigastric vein branches (vessel tags) as the anchoring points of the loop to the chamber (Fig 1, *b*), by sandwiching the tags between the non-porous polycarbonate chamber base and lid when the chamber was closed. The apposed edges of the wound were sutured with 6.0 nylon sutures.

Post-operative treatment. Six hours post-surgery, the rats were injected with saline (Series I) or 0.6 mg/kg weight dose of enoxaparin sodium (Series II) and monitored for post-operative complications (ie, hemorrhage). Thereafter, the rats were injected daily with saline (Series I) or 1.5 mg/kg of enoxaparin sodium (Series II) for the duration of the study.

Anti-factor Xa activity. The in vivo activity of enoxaparin sodium was assessed 1.5 hours after the last enoxaparin sodium administration, in 10 randomly selected treated rats (all time points included) (Series II, n = 6) and control rats (Series I, n = 4). Anesthetized rats had 2 mL of whole blood aspirated via cardiac puncture into a blood tube containing 3.2% buffered trisodium citrate anticoagulant (BD Vacutainer, Franklin Lakes, NJ). The samples were analyzed for anti-factor Xa activity[21] on a STAGO STAR coagulation analyzer.

AVL chamber retrieval, weight, and volume displacement measurements. Two hours prior to chamber retrieval, the rats were injected with saline (Series I) or 1.5 mg/kg enoxaparin sodium (Group II) and then anesthetized with phenobarbitone (30 mg/kg). The constructs

were surgically removed and weight and volume displacement measurements were performed as described previously.[5] The AVL construct was fixed by immersion in 4% paraformaldehyde, cut into 1-mm thick vertical slices, and processed into paraffin for routine histology. Animals were euthanized by intraperitoneal injection of Lethobarb (200 mg/kg, Virbac, Peakhurst, Australia).

Histology. To aid the morphometric counting to determine the absolute volume of fibrin, connective tissue, and blood vessels in the constructs, 3 μm-thick consecutive paraffin sections were dewaxed, hydrated, and stained with hematoxylin and eosin (H&E), Masson's Trichrome stain to identify collagen, Fraser-Lendrum stain to localize fibrin[22] and immunohistochemically with *Bandeiraea Simplicifolia lectin* (Vector Laboratories, Burlingame, Calif) to identify blood vessels.[23] The tissue was examined under a light microscope and photographed with a Zeiss digital camera (Carl Zeiss, Jena, Germany).

Morphometric analysis. To determine the volume of tissue components (AVL, fibrin matrix, connective tissue, and new blood vessels), lectin labeled sections were examined under x20 magnification with a 100 point test grid eyepiece lens placed over the sections. A random microscopic field was selected as a starting point and points falling over the AVL, fibrin matrix, connective tissue, and new blood vessels counted in every fifth field by an observer (Z.L.) blinded to the identity of the tissue. These proportional counts of tissue components were expressed as a percentage of the total points counted (total points representing the whole construct).[24] This measurement is termed the percent volume of a tissue component. To determine the absolute volume of a tissue component, the percent volume of the tissue component was multiplied by the volume displacement of that construct (taken at harvest) to obtain the volume of that particular tissue component.[24]

Statistical analysis. The weight, tissue volume, and anti-factor Xa data are expressed as mean ± SD. Morphometric results are expressed as mean ± SEM of grouped data where n = number of rats per group. For statistical comparison between Series I and II, the means were analyzed using univariate analysis of variance (GraphPad Prism Software, version 4.0; La Jolla, Calif). The results were considered statistically significant when $P < .05$.

For comparison of patency rates between enoxaparin sodium-treated and control Fisher's exact test was used.

RESULTS

Assessment of anti-factor Xa activity. Blood analysis for anti-factor Xa activity showed that the mean value for saline-treated (Series I) rats was 0.26 ± 0.02 IU/mL while for the treated rats (Series II) it was 1.28 ± 0.32 IU/mL. These results indicated that the therapeutic dose of enoxaparin sodium at 1.5 mg/kg was sufficient to inhibit intravascular fibrinogen polymerization.

Animal exclusions and AVL patency rate. Seven rats were excluded from the study, 3 in the control series (1 at 10-days, and 2 at 21-days) and 4 in the treated series (1 at 3 days, 1 at 10 days, and 2 at 21 days) due to infection, AVL occlusion (control groups only), and hematoma formation.

Three days is too early to assess patency in this model. At 10 days, the patency was identical in both groups at 100%. At 21 days, the enoxaparin sodium-treated group maintained 100% patency, whilst the control group had 4 of 6 AVLs patent (67% patency rate). There was no significant difference between the patency rates of the control and enoxaparin sodium -treated groups ($P = .47$, Fisher's Exact test).

Macroscopic appearance. The 3-day control constructs, consisted largely of a fragile gel-like yellow fibrin scaffold surrounding the AVL (Fig 3, *a*). These specimens contained small patches of hemorrhage randomly distributed throughout the fibrin scaffold. In contrast, in the 3-day enoxaparin sodium-treated rats, the AVL constructs appeared smaller and the fibrin scaffold was firm, white, rubbery, and heavily hemorrhagic around the vein graft (Fig 3, *b*).

Ten-day control constructs were characterized by new tissue deposited around the AVL. The fibrin scaffold was predominantly of yellow color with areas of hemorrhage associated with the developing vascularized tissue. Similar observations were made in 10-day enoxaparin sodium-treated constructs, except that these constructs were more hemorrhagic and the fibrin scaffold was white and rubbery. By 21 days, the majority of control constructs contained residual fibrin scaffold at the periphery of the construct (Fig 3, *c*), whereas the enoxaparin sodium-treated constructs were smaller and appeared quite hemorrhagic with little fibrin visible at the periphery of the construct (Fig 3, *d*).

Tissue weight and volume. The inhibition of fibrin polymerization by enoxaparin sodium resulted in the development of smaller constructs by weight and volume compared to control at all time points (Fig 4, *a, b*). At 10 days, enoxaparin sodium-treated constructs were significantly smaller by weight (0.228 ± 0.048 g compared to the control group: 0.337 ± 0.016 g, $P < .001$) and volume (0.184 ± 0.039 mL compared to the control group: 0.317 ± 0.015 mL, $P < .01$). These differences persisted at 21 days with weight (0.198 ± 0.043 g compared to the control group: 0.306 ± 0.053 g, $P < .01$) and volume of constructs (0.148 ± 0.041 mL compared to the control group: 0.285 ± 0.047 mL, $P < .01$) (Fig 4, *a, b*).

Morphology. At 3 days, the control AVL constructs were characterized by formation of a fibrin exudate around the AVL (Fig 5, *a*). The fibrin scaffold consisted of long uniform fibers and was deposited in layers in alternate arrangements of thin wispy strands of fibrin and slightly more dense areas of fibrin, suggesting gradual deposition. The fibrin matrix was infiltrated with inflammatory cells and early vessel sprouts in three constructs. Spindle-shaped cells were observed in close vicinity to the vein wall (Fig 5, *c*), but were also scattered throughout the fibrin scaffold. These cells were observed either alone or in contact with another "like" cell.

In 3-day enoxaparin sodium-treated animals, the spindle-shaped mesenchymal cells were observed only in the areas

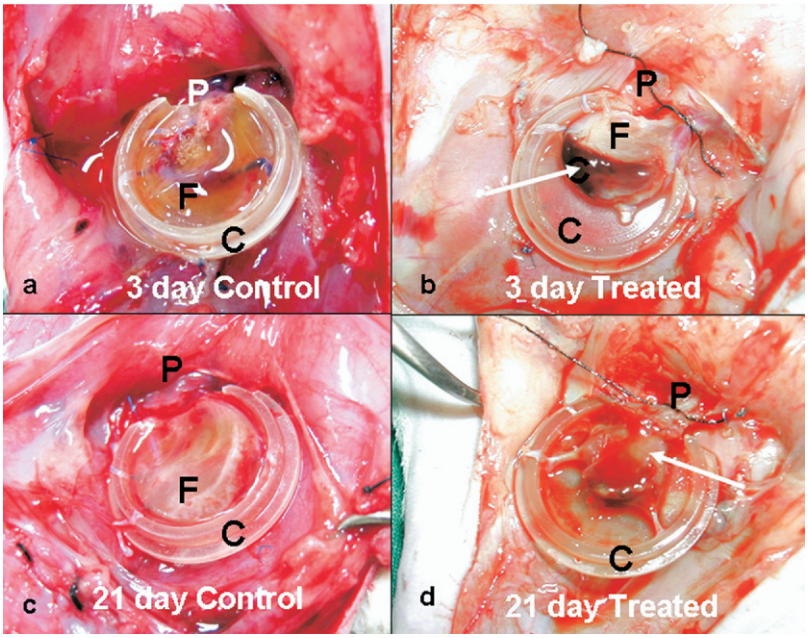

Fig 3. Macroscopic view of control and enoxaparin sodium–treated constructs at harvest. **a,** Three-day control constructs. Note that the major part of the construct consists of translucent yellow fibrin (F). **b,** Three- day enoxaparin sodium-treated construct. Note that the construct is smaller than the control (*a*) and the fibrin matrix includes a large amount of blood (*arrow*). **c,** Twenty-one-day control construct. Note that the outer surface of the construct still demonstrates a fibrin layer (*F*). **d,** Twenty-one-day enoxaparin sodium-treated construct. Note that the outer surface does not demonstrate fibrin, but rather more solid connective tissue (*arrow*). Haemorrhage also covers the construct surface. *P,* pedicle (femoral artery and vein at chamber entrance); *C,* chamber base.

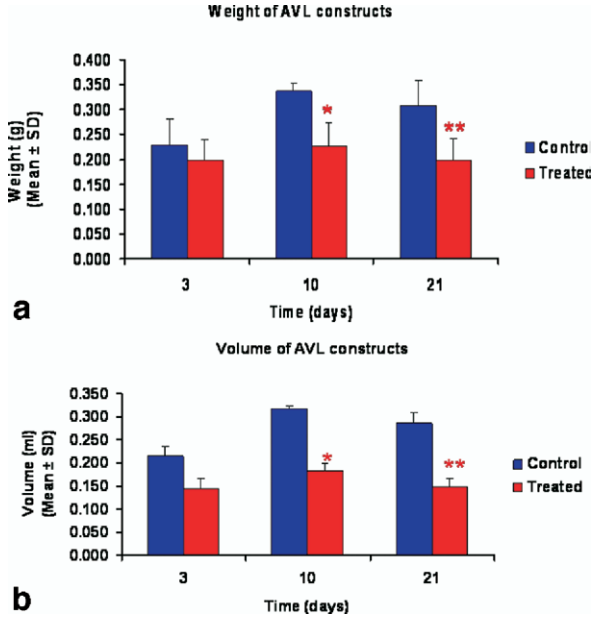

Fig 4. a, Weight and **b,** volume of control and enoxaparin sodium-treated constructs at 3, 10, and 21 days. At 10 days (*) enoxaparin sodium-treated constructs were significantly smaller by weight ($P < .001$) and volume ($P < .01$). These significant reductions persisted at 21 days (**) for both weight ($P < .01$) and volume ($P < .01$).

immediately adjacent to the AVL. Also, in contrast to control constructs, fibrin strands appeared as fragmented and short (Fig 5, *d*). The major histological difference between control and enoxaparin sodium-treated constructs was the presence of significant hemorrhage and inflammation in the fibrin scaffold of treated constructs (Fig 5, *b*, *d*). None of the enoxaparin sodium-treated constructs showed signs of thrombus formation in the AVL or demonstrated capillary sprouting.

In 10-day control constructs different stages (zones) of construct development were evident (Fig 6, *a*, *c*). The innermost zone was the maturing vascularized connective tissue that formed a cuff around the AVL adjacent to the AVL adventitial surface. External to this mature zone was a prominent, wide capillary proliferative zone completely surrounding the tissue cuff. External to the proliferative zone was a thin layer of hemorrhage associated with hyperpermeable vessels which was part of the most peripheral zone of the construct, the fibrin scaffold. New blood vessels within the mature zone and proliferative zone were clearly perfused with blood in all specimens. The proliferative zone was 'invading' the extensive fibrin scaffold associated with the periphery of the specimen adjacent to the chamber. The first arterioles were observed close to the AVL in all control constructs.

In 10-day enoxaparin sodium-treated constructs (Fig 6, *c*, *d*, *e*), most of the fibrin was replaced by vascularized

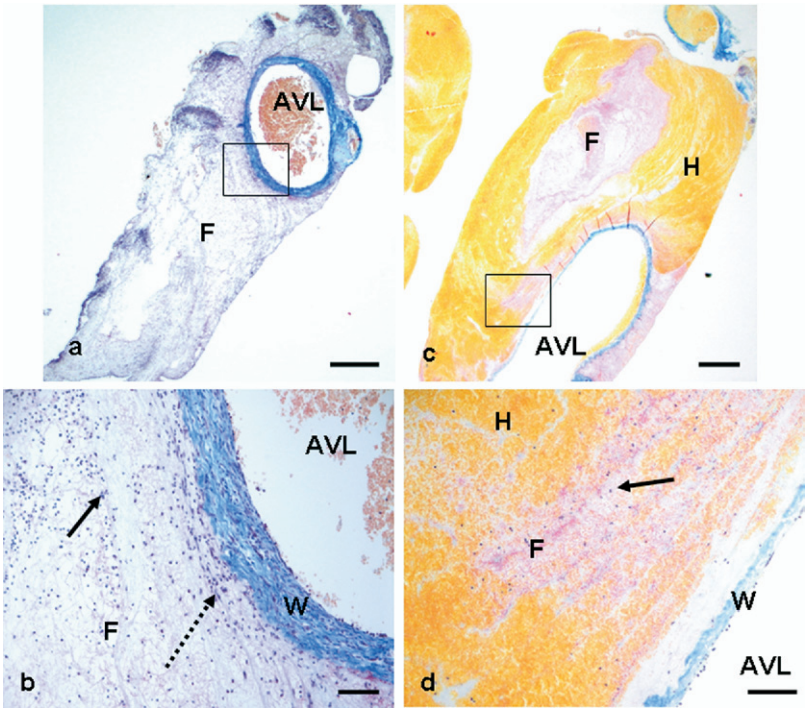

Fig 5. Three-day control and enoxaparin sodium–treated constructs. (Fraser-Lendrum stain). **a,** Three-day control. The AVL is surrounded by an extensive fibrin exudate (purple area). **b,** Higher power micrograph of three day control [area within box in (*a*)] demonstrating the arteriovenous wall (*W,* region of femoral vein) surrounded by fibrin exudate (*F*) showing as purple fibers, infiltrated by inflammatory cells (blue nuclei, *arrow*). Also evident are mesenchymal cells close to the AVL wall (dotted arrow). **c,** Three-day enoxaparin sodium-treated construct showing relatively small areas of fibrin (*F,* pinkish material) and larger areas of haemorrhage (*H,* yellow areas). **d,** Higher power micrograph of three-day enoxaparin sodium-treated construct [area within box in (*c*)] demonstrating the arteriovenous wall (*W,* region of vein graft) surrounded by areas of fragmented fibrin (*F,* and *dotted arrow*) and larger areas of haemorrhage (*H,* yellow). Occasional inflammatory cells are present (*arrow*). Scale bars (*a* and *c*) = 500 μm, (*b* and *d*) = 100 μm.

connective tissue. A thin capillary proliferative zone was present close to the external boundary of the construct, surrounded by a narrow area of hemorrhagic fibrin scaffold (Fig 6, *d, e*). Only two of six treated constructs demonstrated arteriolar development at 10 days.

In 21 day control constructs the fibrin scaffold was present at the periphery of the construct surrounding a prominent capillary proliferative zone, characterized by immature hyperpermeable vessels (Fig 7, *a, c*). The fibrin scaffold continued to act as a provisional matrix for cellular migration from the proliferative zone.

In 21-day enoxaparin sodium-treated constructs, the fibrin scaffold was almost non-existent. There was no capillary proliferative zone and the majority of specimens appeared to have reached their full development prior to 21 days (Fig 7, *c, d, e*).

Morphometry: absolute volume of fibrin, connective tissue, and new blood vessels. Absolute fibrin volume decreased gradually over time in control and enoxaparin sodium-treated constructs (Fig 8, *a*). Absolute fibrin volume was always less, but not significantly so in the enoxaparin sodium-treated groups at each time point. The 10-day time point difference neared significance ($P = .058$).

The absolute volume of new connective tissue increased from 3 to 10 days in both control and enoxaparin sodium-treated constructs (Fig 8, *b*) and stabilized thereafter. The enoxaparin sodium-treated constructs demonstrated a reduced connective tissue volume compared to control at all time points, although the differences were not significant (Fig 8, *b*).

In 3-day control constructs, angiogenesis had commenced and vascular volume was small (0.0004 mL), whilst in enoxaparin sodium-treated constructs angiogenesis was not evident. Throughout the study period, absolute vascular volume was decreased in enoxaparin sodium-treated constructs (Fig 8, *c*) compared to controls, and was significantly different at 10 days (0.012 ± 0.002 mL for treated vs 0.029 ± 0.03 mL for controls, $P < .05$).

Definite trends were observed in the diminished volume of fibrin, connective tissue, and new blood vessels in all enoxaparin sodium treated groups compared to control, with only the 10-day vascular volume being significantly different. It is possible that a Type II error has occurred and

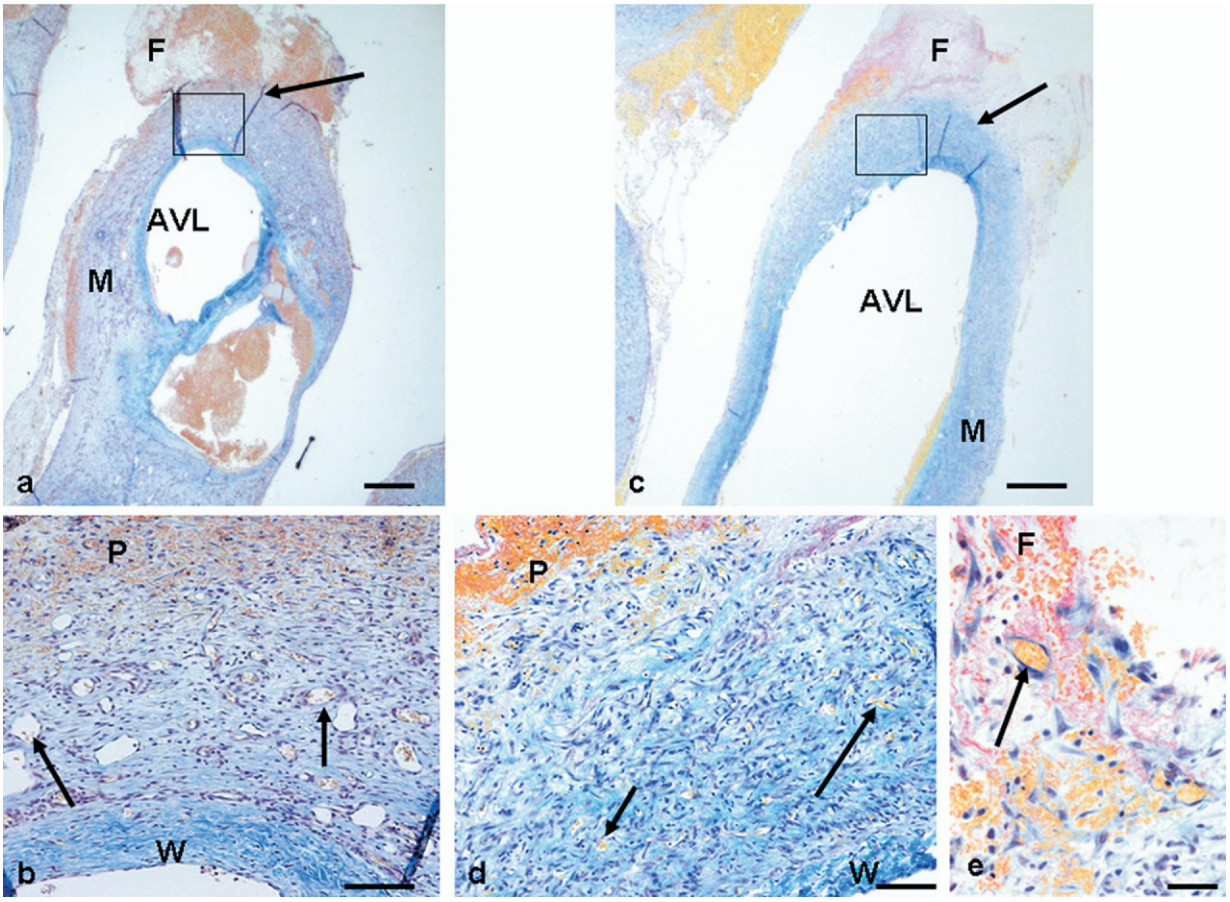

Fig 6. Ten-day control and enoxaparin sodium–treated constructs. (Fraser-Lendrum stain). **a,** Ten-day control and **(b)** enoxaparin sodium–treated constructs both demonstrating a central AVL surrounded by maturing blood vessels in connective tissue (*M*) and fibrin (*F*) external to the new blood vessels. The arrow indicates the position of the proliferative zone. Scale bars = 500 μm. **b, and d,** Higher power of maturing tissue from 10-day control **(b)** and enoxaparin sodium–treated constructs **(d)** [areas in boxes in **(a)** and **(c)**] demonstrating the AVL wall (W) and mature capillaries (*arrows*) in collagenous connective tissue. External to the mature tissue is the proliferative zone (*P*). Scale bars = 100 μm. **e,** Higher magnification of tissue edge of treated construct showing a new capillary (*arrow*) growing into the external fibrin (*F*) scaffold. Scale bar = 20 μm.

larger animal numbers in each group would have produced significance.

DISCUSSION

During wound healing or hemostatic imbalances, a complex cascade of events leads to polymerization of soluble fibrinogen, a 340 kDa glycoprotein present in high concentrations in the circulating blood, by thrombin into the insoluble, cross-linked fibrin clot.[25] In this study, we report that a fibrin scaffold, formed through normal physiological responses following surgical formation of an AVL and protected by a chamber space, has a pivotal role in the development of a surgically generated tissue engineered microcirculatory network in vivo. Furthermore, the quality and volume of deposited fibrin determines the whole construct size, the total tissue volume, and rate of maturation of new blood vessels in the construct.

Early deposited fibrin in this model provides a three-dimensional scaffold that supports migration of inflammatory cells, vascular precursors and myofibroblasts, and the formation of a new vascular network.[5] These events are likely to be controlled by the presentation of cytokines and growth factors, bound to fibrin, and available immediately upon cleavage to the migrating cells,[6,7] and, cell integrin-fibrin matrix interaction.[26,27] For example, the cellular recognition of the Arg-Gly-Asp (RGD) peptide in fibronectin is mediated by the $\alpha_v\beta_3$ integrin, resulting in the activation of various signaling pathways governing cellular shape, migration, and function.[28,29] Subsequently many synthetic matrices have been modified to include the RGD sequence on their surface[28] to reflect fibrin structure and generate a tissue construct with specific cellular composition and possibly a reduced foreign body response to the synthetic part of the hybrid scaffold.[28,30]

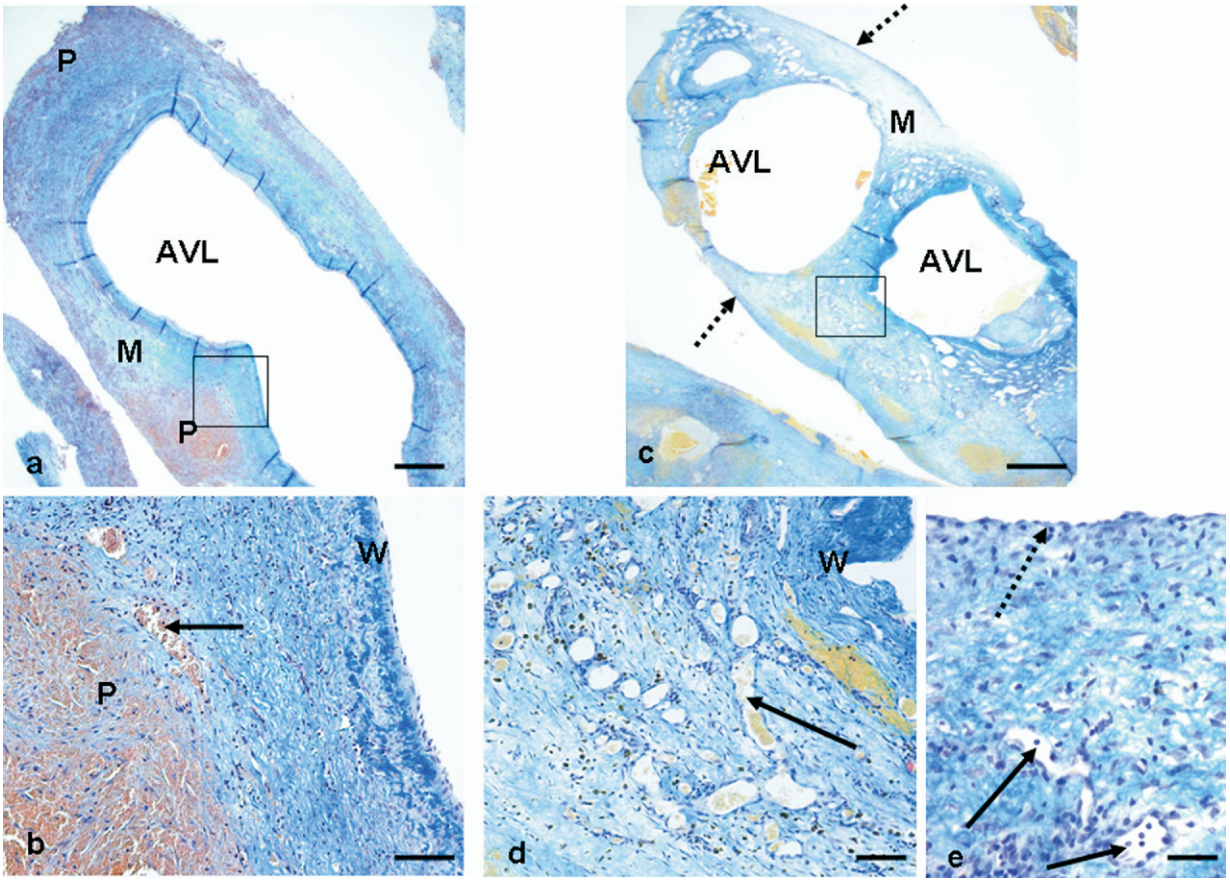

Fig 7. Twenty one-day control and enoxaparin sodium–treated constructs. (Fraser-Lendrum stain). **a,** Twenty one-day control and **(c)** enoxaparin sodium–treated constructs with a central AVL surrounded by largely mature construct tissue (*M*). In treated constructs **(c)** the new tissue has grown to the edge of the fibrin scaffold (*dotted arrows*) and the proliferative zone is absent. In the control **(a)** the proliferative zone (*P*) persisted indicating that the construct was still growing. The fibrin zone was present at the periphery of the specimens. Scale bars = 500 μm. **b, and d,** Higher power of twenty one-day control **(b)** and enoxaparin sodium–treated constructs **(d)** [areas in boxes in **(a)** and **(c)**] demonstrating the AVL wall (*W*) and mature capillaries (*arrows*) in collagenous connective tissue. The proliferative zone (*P*) was seen external to the mature tissue only in the control specimens. Scale bars = 100 μm. **e,** Higher magnification of tissue edge of treated construct demonstrating no fibrin scaffold or proliferative zone. Mature connective tissue comes to the edge of the construct (*dotted arrow*, compare with 4e). Solid arrows: maturing capillaries. Scale bar = 20 μm.

The inhibition of fibrin matrix polymerization by enoxaparin sodium in AVL constructs has resulted in reduced fibrin deposition in the chamber space and changes in fibrin quality, apparent histologically as fragmented fibrin strands. This effect was also evident in the form, of a white, rubbery macroscopic appearance of the fibrin in the treated constructs vs yellow, gel-like fibrin scaffold in the control constructs. This change in physical properties of the fibrin scaffold and the delayed migratory pattern of mesenchymal and endothelial precursor cells suggests that the structure and integrity of the fibrin is important in the development of vascularized connective tissue in this model. These findings support in vitro studies showing that the polymerization of fibrin under different pH affects the in-growth of capillary tubes into the fibrin matrix[31,32] while the addition of low molecular weight heparin during polymerization reduces the in-growth of capillary tubes into fibrin matrix.[33]

There are a number of studies in the literature which suggest that low molecular weight heparins inhibit angiogenesis[34] although not all studies support this concept.[35]

It has also been demonstrated by Collen et al[33] that low molecular weight heparins significantly inhibit microvascular in-growth into a fibrous stroma, by altering the fibrin matrix formed so that it is a less favorable environment for microvascular invasion. Our study supports this later hypothesis as distinct alterations in the fibrin matrix quality were observed macroscopically and microscopically in the treated group. Although the angiogenic process appeared similar in both treated and control groups of our study, the

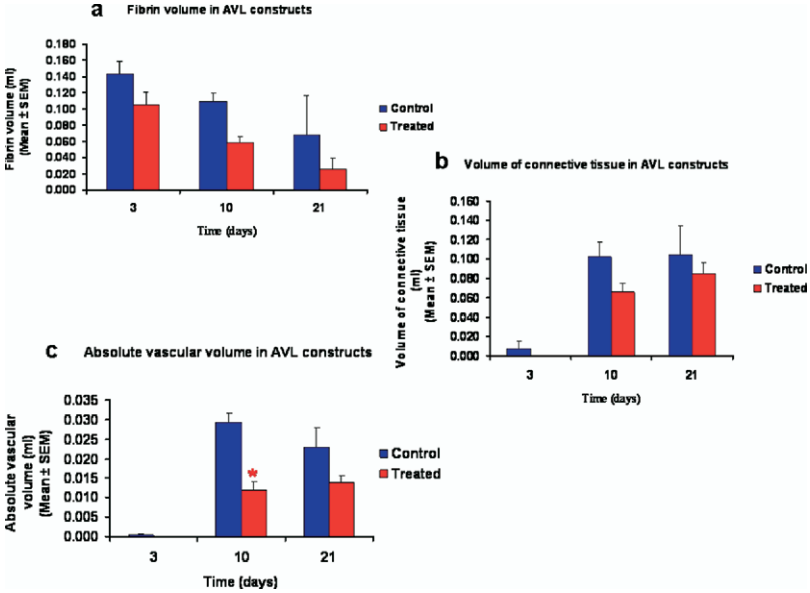

Fig 8. Morphometry. **a,** Absolute volume of fibrin in control and enoxaparin sodium–treated constructs demonstrating reduced fibrin volumes in treated constructs throughout the study. **b,** Absolute volume of new connective tissue in controls and enoxaparin sodium–treated constructs, demonstrating lower volumes throughout the study in treated constructs. **c,** Throughout the study period absolute vascular volume was decreased in enoxaparin sodium-treated constructs. This difference was statistically significant at 10 days (*), 0.012 ± 0.002 mL for treated vs 0.029 ± 0.03 mL for controls, $P < .05$.

migration of endothelial precursors was delayed in the treated group, as too was the onset of the first microvascular tube formation (capillary formation), and arteriole formation. In addition, the reduced fibrin volume resulting from low molecular weight heparin treatment reduced the volume of matrix available for microvascular invasion, and therefore reduced the time over which angiogenesis took place and ultimately significantly reduced the weight and volume of low molecular weight heparin (enoxaparin sodium) treated constructs.

In this study, the enoxaparin sodium-induced fragmentation of fibrin matrix was associated with delayed cellular migration and angiogenesis but was insufficient to completely prevent the development of vascularized connective tissue. The delayed angiogenic development was evident by the absence of new blood vessels in the treated constructs at 3 days, while angiogenic sprouting was present in 3 out of 6 control constructs at 3 days. At 10 days, the treated constructs had a smaller capillary proliferative zone, a significantly reduced vascular volume, and delayed arteriolar development compared to controls. While these results further support the hypothesis that the quality and volume of the polymerized fibrin scaffold is pivotal in the development of the new blood vessels as well as the determinant of construct size, vascular volume, and rate of vascular maturation in AVL constructs, it cannot be excluded that low molecular weight heparin enoxaparin sodium might have a detrimental effect on microvascular endothelial cell proliferation[34] subsequently leading to a reduced vascular volume as reported here. A statistical difference in absolute vascular volume was not observed at 21 days, and is most likely due to partial microcirculatory remodeling already taking place in the 21-day control constructs.[5]

The study reported here has demonstrated that in treated 21-day constructs when no fibrin scaffold was present, the proliferative zone no longer existed indicating that the vascular and tissue growth ceased prior to 21 days in the treated group. In contrast, in 21-day controls, where an intact fibrin matrix was still present, angiogenesis continued as evident by a presence of a persistent proliferative zone indicating a further growth potential. Therefore, AVL construct development is limited by the availability of the fibrin scaffold deposited around the AVL i.e. decreased fibrin matrix deposition around the AVL results in decreased volume of generated vascularized tissue.

Although the concentration of enoxaparin sodium used in this study was sufficient to reduce intravascular fibrinogen polymerization, a complete inhibition of extravascular fibrin formation around the AVL was not achieved. This suggests that enoxaparin sodium might not act extravascularly in the same manner as intra-vascularly either because a significant amount of active extra-vascular profibrotic factors are present in the deposited scaffold (but not in the blood), or alternatively, once extra-vascular, enoxaparin sodium was unable to bind to anti-thrombin III and completely inhibit thrombin generation. This would subsequently lead to activation of factor X and fibrin polymerization once the factor reached the extra-luminal surface of the AVL.

No histological evidence of AVL occlusion was observed in the enoxaparin sodium-treated constructs indicating that use of low molecular weight heparins such as enoxaparin sodium will decrease the AVL occlusion rate otherwise observed in a series extending to 16 weeks at 15.1% of all untreated AVL constructs[5] and in this study to a decline in untreated AVL construct patency from 100% at 10 days to 67% at 21 days. However, the use of enoxaparin sodium as an anticoagulant would result in generation of smaller AVL constructs which should be taken into consideration if a large three-dimensional tissue or organ is required.

We have previously noted that occlusion of the AVL does not in fact lead to an adverse outcome, but rather prolongs angiogenesis and increases construct size if occlusion occurs after approximately one week.[3] The AVL chamber model has considerable plasticity and AVL occlusion after 7 days has no adverse effect on the new microcirculatory bed or supporting connective tissue and is, therefore, not a significant issue in this model.

An alternative to in vivo generation of fibrin matrix are the commercially available fibrin sealants which are composed of high concentrations of plasma derived complexes of purified pathogen inactivated human fibrinogen and thrombin.[36] Fibrin sealants are semi-rigid, water-resistant polymers which are relatively inert. This inertness is designed to reduce inflammation, cellular migration, foreign body reaction, and cellular necrosis associated with the use of fibrin sealants.[37,38] However, the ability to support the development of a vascularized connective tissue construct seems to be dependent of the concentration of the fibrin present in the sealant mixture. For example Cassell et al have shown that the use of commercial fibrin preparations at 20 and 80 mg/mL resulted in the development of smaller tissue constructs with decreased vascular volume.[39] Similar observations have been reported by Polykandriotis et al where a fibrin sealant used at 30 mg/mL resulted in delayed angiogenesis.[40] However, Arkudas et al[41] have shown that both VEGF and basic fibroblast growth factor can be incorporated into this fibrin sealant (10 mg/mL of fibrin, 2 IU/mL of thrombin and 1500 KIE/mL aprotinin) with a resulting increased vascular density at 2 weeks compared to constructs containing the same fibrin sealant matrix without growth factors. Better understanding of biochemical properties of fibrin and cell-fibrin scaffold interaction should enable further modifications of the chemical composition of fibrin-based products that may improve their use in tissue engineering.

CONCLUSION

The volume and integrity of fibrin matrix in AVL constructs acts as a limiting factor in AVL construct development since:

- Fibrin matrix provides a provisional scaffold for cellular migration and connective tissue deposition. Changes in fibrin polymerization alter the pattern of cellular migration, delay angiogenesis, and arteriole maturation.
- A decrease in fibrin polymerization and the subsequent reduced volume of deposited fibrin in the chamber significantly reduces the volume and weight of tissue constructs at 10 and 21 days, and significantly reduces absolute vascular volume at 10 days.

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AUTHOR CONTRIBUTIONS

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Writing the article: ZL, JT, GM
Critical revision of the article: ZL, JT, WM, EK, GM
Final approval of the article: ZL, JT, WM, ET, GM
Statistical analysis: ZL, GM
Obtained funding: ZL, WM, ET
Overall responsibility: GM

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